Exhibit 1

The NH₂-terminal region of the active domain of sonic hedgehog is necessary for its signal transduction

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Abstract The NH₂-terminal domain of sonic hedgehog (residue 25–198) was expressed in both yeast and animal cells. The yeast-derived NH₂-terminal domain of sonic hedgehog was less active by far than the animal cell-derived counterpart. The yeast-derived NH₂-terminal domain of sonic hedgehog lacked 10 amino acids from the NH₂-terminus. This cleavage of the yeast-derived NH₂-terminal domain of sonic hedgehog might due to Kex 2. In contrast, a mutant yeast-derived NH₂-terminal domain of sonic hedgehog (Lys-33 to Thr) retained its NH₂-terminus and its activity was comparable to that of the animal cell-derived NH₂-terminal domain of sonic hedgehog. The NH₂-terminal deleted NH₂-terminal domain of sonic hedgehog completely lost its activity, nevertheless it inhibited the alkaline phosphatase activity induced by the animal cell-derived NH₂-terminal domain of sonic hedgehog in a dose-dependent manner. These data suggest that the NH₂-terminal deleted NH₂-terminal domain of sonic hedgehog retains a receptor-binding ability and that the NH₂-terminal peptide of the NH₂-terminal domain of sonic hedgehog is necessary for its signal transduction.
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Key words: Alkaline phosphatase; Antagonist; Signal transduction; Sonic hedgehog

1. Introduction

The proteins of the hedgehog family are important signalling proteins that regulate various aspects of the animal development [1–3]. Sonic hedgehog (Shh) in vertebrates is expressed in embryonic cells, resulting in patterning and modulation of differentiation such as a polarizing activity in the posterior limb bud, the notochord, and the floor plate of the neural tube [4–9]. Several studies also have suggested that Shh might have osteo(chondro)genic [10–12] or tumorigenic effects [13–16]. Although the molecules that are involved in hedgehog signalling, such as putative receptor patched (Ptc) [17,18], signal transducer smoothened (Smo) [18,19] and transcription factor Gli [20,21], have recently been elucidated [22,23], the signalling mechanism action of Shh is not completely understood.

Concerning molecular aspects, Shh (45 kDa) is an inactive precursor protein which converts itself into an active molecule, 20 kDa NH₂-terminal domain of sonic hedgehog (Shh-

N, residue 25–198), through autocatalytic internal cleavage [24–26], followed by cholesterol attachment at its COOH-terminal [27,28]. Most of Shh-N processed from Shh (45 kDa) undergoes cholesterol modification and remains in the cell membrane in bound form, while a small amount of unmodified Shh-N is secreted in a soluble form [27]. Therefore, Shh-N signalling seems to occur by cell to cell interaction. However, recombinant Shh-N expressed in the soluble form has a comparable biological activity to the bound form [24–32]. In the present paper, we expressed mouse Shh-N in a mammalian cell line and in yeast and observed the loss of activity in yeast, as judged by the induction of alkaline phosphatase (ALPase) activity, in mouse mesenchymal cell line C3H10T1/2 cells. These cells have previously been shown to express Ptc [17] and respond to hedgehog proteins [12,33]. In characterizing this protein, we showed that the NH₂-terminal region of Shh-N played an important role in receptor activation in the hedgehog signalling mechanism.

2. Materials and methods

2.1. Plasmid construction and expression of Shh-N in L-cells

cDNA encoding the NH₂-terminal domain of mouse sonic hedgehog (amino acid positions 1–198) was amplified by PCR from the full length mouse Shh cDNA, provided by Dr McMahon, Harvard University, and cloned into the pABWN vector provided by Dr Miyazaki, Tokyo University. The resulting plasmid was transfected into mouse fibroblastic L-cells (Riken Cell Bank, Saitama, Japan) using lipofectamin reagent (Gibco BRL, Gaithersburg, MD, USA). These transfectants carrying high number of copies of expression plasmids were selected with 800 μg/ml G418 (Gibco BRL) in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with 10% fetal bovine serum (FBS, purchased from Gibco BRL). The transfectants were cultured in DMEM supplemented with 0.5% FBS and 800 μg/ml G418 for 3 or 4 days and the culture supernatants were collected by centrifugation at 3000 rpm for 10 min and then filtered through a 0.22 μm filter.

2.2. Expression of Shh-N in yeast

To construct an expression vector of mouse Shh (amino acid positions 25–198), the cDNA was amplified by PCR with a primer set consisting of 5'-TCTCTCGAGAAAAGATGTGGGCCCGGCAGG-GGGTTTGGAAA-3' and 5'-GAATTCAGCCGCCGGATTTGGC-CGCCACGGA-3' and the product was cloned into a pPIC9K vector within *Xho*I and *Eco*RI sites (Invitrogen, CA, USA). To substitute the 33rd amino acid of Lys in mouse Shh for that of Thr in Cynops (Japanese newt), the clone was amplified with another set of primers, 5'-TGTGGGCCCGGCAGGGGGGTTTGGAAC-GAGGCGGCA-3' and 5'-GAATTCAGCCGCCGGATTTGGCCG-CCACGGA-3', and the product was subcloned into the same vector at *Apa*I and *Eco*RI sites. Transformants were obtained by introducing each vector into spheroplasts of *Pichia pastoris* KM71 (*arg4 his4 aox1::ARG4*), followed by selection on YPD agar plates (Difco Laboratories, MI, USA) with 0.5–20 mg/ml G418. To express the protein, the clones were pre-cultured in BMG liquid media (Difco Laboratories) at 30°C for 18 h, transferred to BMMY media (Difco Labora-

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tories) to give an $A_{650}=1.0$ and induced by the addition of methanol to a final concentration of 0.5% every 24 h according to the instruction manual of Invitrogen. Cells were harvested 48 h after induction.

2.3. Purification of recombinant Shh-N expressed in L-cells and yeast?

The culture supernatants were applied to a HiTrap SP column (Amersham Pharmacia biotech, Uppsala, Sweden), previously equilibrated with 20 mM sodium phosphate buffer (PB), pH 7.4, containing 50 mM NaCl. After washing the column with the same buffer, the proteins were eluted with 1 M NaCl in PB, pH 7.4. Eluates of the SP column were further purified by gel filtration using Superdex 200 pg, 1.6×60 cm (Amersham Pharmacia biotech) with PB containing 150 mM NaCl. The fractions containing Shh-N were pooled and concentrated with Centriprep-10 (Amicon, MA, USA). The protein concentration was determined by the BCA protein assay kit (Pierce, IL, USA).

2.4. SDS-PAGE and Western blot analysis

SDS-PAGE was performed with a 15–25% gradient gel (Daiichi pure chemicals, Tokyo, Japan). Proteins were visualized with silver-stain kit (Daiichi pure chemicals) or Coomassie brilliant blue. Western blotting was performed with a purified rabbit IgG fraction raized against Shh peptide (amino acid 143–157). Detection of Shh-N was carried out with an immunostaining kit (Konica, Tokyo, Japan) with horseradish peroxidase-conjugated swine anti-rabbit IgG (DAKO, Glostrup, Denmark) as a secondary antibody.

2.5. Determination of the amino-terminal amino acid sequence

Amino-terminal amino acid sequence analysis was performed by a 476A Protein Sequencer (Perkin-Elmer, CT, USA).

2.6. Measurement of the alkaline phosphatase activity

This assay was performed using a pluripotent mouse fibroblastic cell line C3H10T1/2 (purchased from ATCC). The cells were seeded into 24 well plates (10000 cells/well) and grown in BME (Gibco BRL) supplemented with 10% FBS (Gibco BRL). After 3 days culture, the cells were rinsed with serum-free medium once, then the medium was replaced with 2% FBS containing test samples, followed by a further incubation for 3 days. For the measurement of ALPase activity, cells were washed twice with PBS and cell layers were extracted with 200 μl of Lysis Buffer (0.2% NP-40, 1 mM MgCl₂). The cell extract was transferred to a microtube and centrifuged for 5 min at 10000 rpm. The enzyme activity in the supernatant was assayed with 10 mM (final concentration) p-nitrophenyl phosphate (WAKO pure chemicals, Osaka, Japan) as a substrate in 0.1 M glycine buffer, pH 10.4, containing 1 mM ZnCl₂ and 1 mM MgCl₂. The increase in absorbance at 405 nm was monitored every 2 min over 40 min and the ALPase activity (nmol p-nitrophenol (pNp) produced/min) was calculated from a linear range. The protein content in each sample was determined with the BCA protein assay kit (Pierce) using bovine serum albumin as a standard sample. The specific activity of ALPase was given as nmol pNp/min/mg protein.
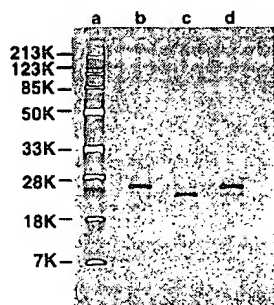

Fig. 1. SDS-PAGE of purified recombinant Shh-Ns expressed in L-cells and yeast. Each sample (100 ng) was loaded onto a 15–25% polyacrylamide gradient gel. Proteins were visualized with silver-staining (lane a, Bio-Rad, pre-stained molecular weight marker; lane b, L-Shh-N; lane c, Y-Shh-N; lane d, MuY-Shh-N).
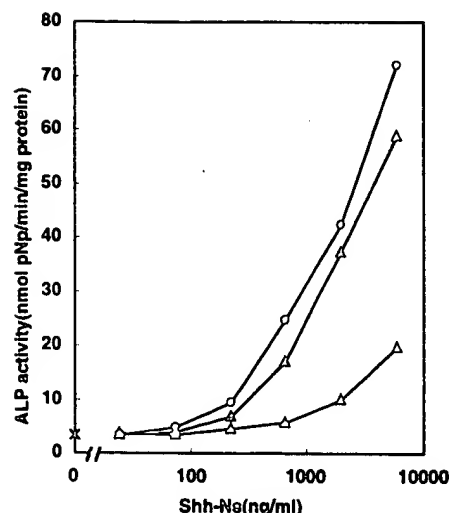

Fig. 2. Induction of ALPase activity by Shh-Ns in C3H10T1/2 cells. The ALPase activity induced by various amounts of Shh-Ns was measured. L-Shh-N(○), Y-Shh-N (▲), Mutant Y-Shh-N (△) and control (x).

2.7. Limiting digestion of Shh-N by trypsin

The animal cell-derived NH₂-terminal domain of sonic hedgehog (L-Shh-N) (about 300 μg/ml) in PB containing 150 mM NaCl was mixed with an equivolume of various dilutions of immobilized trypsin agarose gel (Pierce) for 5 min at 20°C and the digests were obtained by centrifugation as supernatant.

3. Results

Shh-Ns expressed in L-cells and yeast were purified by the same method. On SDS-PAGE analysis under reducing conditions, the purified L-Shh-N migrated as a single band with an apparent mass of 24 kDa as shown in Fig. 1. In this paper, the 24 kDa protein corresponds to Shh-N (residues 25–198), which is described as a 20 kDa protein in many papers. This might be due to a difference in the molecular weight marker used. On the other hand, the purified yeast-derived NH₂-terminal domain of sonic hedgehog (Y-Shh-N) consisted of more than 90% of a different molecular mass (22 kDa) protein (Fig. 1). Immunochemical analysis revealed that both proteins existed during expression in yeast and reacted with rabbit polyclonal antibodies to the Shh-N peptide (residues 143–157) (data not shown). An amino-terminal sequence analysis showed that the NH₂-terminal amino acid of L-Shh-N was Cys-25 only, but that of Y-Shh-N was mainly Arg-35 besides Cys-25. This deletion of 10 amino acid peptides from the NH₂-terminal seems to be caused by proteolysis at the COOH-terminal of a dibasic amino acid sequence (Lys-33, Arg-34) by a processing enzyme of yeast, Kex 2. Therefore, we converted a basic amino acid (Lys-33) to a neutral one, Thr, and expressed it in yeast. Most of the mutant Y-Shh-N (MuY-Shh-N) was secreted as a 24 kDa protein, having Cys-25 at the NH₂-terminus. SDS-PAGE of the purified MuY-Shh-N is also shown in Fig. 1.

Biological activities of L-Shh-N, MuY-Shh-N and Y-Shh-N were compared by examining the ALPase-inducing activity in C3H10T1/2 cells (Fig. 2). L-Shh-N and MuY-Shh-N showed almost the same activity, while Y-Shh-N showed less activity.

This indicates that the lack of 10 amino acid residues NH$_2$-terminal of Shh-N results in a loss of activity.

To determine whether the decreased activity of Y-Shh-N was due to a NH$_2$-terminal deletion or not, samples which contained NH$_2$-terminal deleted L-Shh-N in varying amounts were produced by trypsin digestion and subjected to measurement of the biological activity (Fig. 3). Increasing the ratio of trypsin to L-Shh-N resulted in the production of a 22 kDa protein with little of the further digested 21 kDa protein (Fig. 3a) and a decrease of the ALPase-inducing activity (Fig. 3b). After complete loss of the 24 kDa protein (Fig. 3a, lane g), the digests were subjected to amino-terminal sequence analysis. The data showed that the NH$_2$-termini of the 22 kDa protein were Arg-34 (40%) and Arg-35 (40%), and Lys-39 (20%) for the 21 kDa protein. The amino acid composition of the digest agreed well with the theoretical values of the peptide from Arg-35 to Lys-195 of Shh-N, indicating a deletion of three
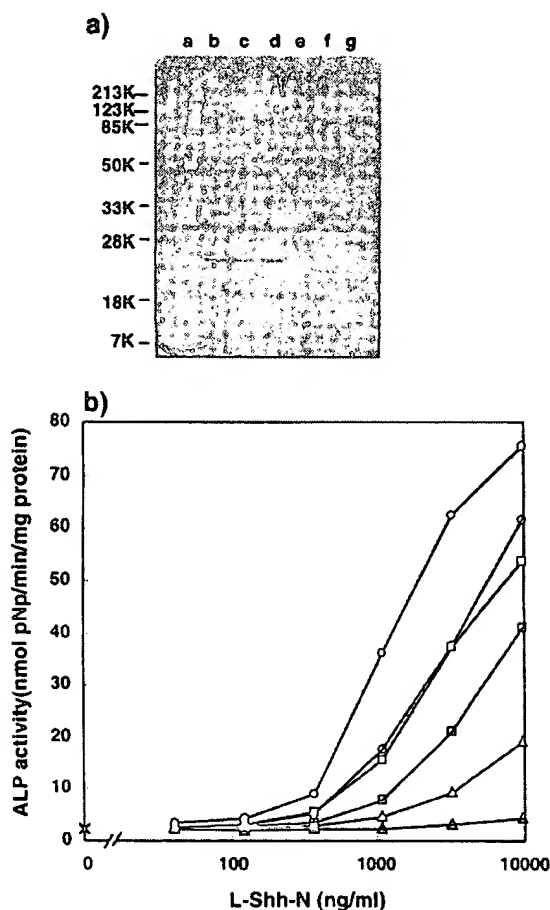

Fig. 3. Loss of ALPase-inducing activity of L-Shh-N following release of its NH$_2$-terminal peptide with trypsin. L-Shh-N was treated by various dilutions of immobilized trypsin agarose gel. 5 min later, the supernatant was collected by centrifugation. (A) 10 μl of each sample was loaded onto a 15–25% polyacrylamide gradient gel. Proteins were visualized with Coomassie brilliant blue (lane a, Bio-Rad, pre-stained molecular weight marker; lane b, L-Shh-N; lanes c–g, L-Shh-N treated by various dilutions of immobilized trypsin agarose gel, ×100, ×50, ×20, ×10 and ×5, respectively. (B) ALPase-inducing activity of each L-Shh-N was determined in C3H10T1/2 cells. L-Shh-N was incubated with ×100 (○), ×50 (●), ×20 (△), ×10 (▲) and ×5 (□) dilutions of immobilized trypsin agarose gel.
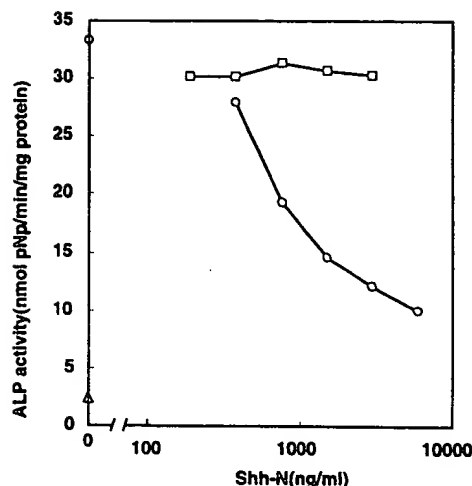

Fig. 4. Inhibitory effects of desN-Shh-N on the ALPase activity induced by L-Shh-N. C3H10T1/2 cells were incubated with (●) or without (○) a serial 3-fold dilution of desN-Shh-N for 30 min before addition of 600 ng/ml of L-Shh-N. ALPase induction was then performed for 3 days.

amino acids at the COOH-terminal of Shh-N. These results suggest that the loss of ALPase-inducing activity of Shh-N in C3H10T1/2 cells is related to a deletion of its NH$_2$-terminal region.

We also isolated 22 kDa/21 kDa protein, desN-Shh-N, using a SP-Sepharose column or a reverse-phase HPLC column from the sample produced by the same condition in Fig. 3a, lane g, and its ALPase-inducing activity was measured. There was no activity even with 6000 ng/ml of desN-Shh-N and desN-Shh-N inhibited the ALPase-inducing activity by L-Shh-N in a dose-dependent manner (Fig. 4). In contrast, denatured L-Shh-N by treatment with 6 M guanidine-HCl had neither an ALPase-inducing activity nor inhibitory activity. These data suggest that desN-Shh-N retained the ability to bind a receptor but lost the ability to follow signal transduction on the receptors. It can be considered that nine (or 10) NH$_2$-terminal amino acid peptides of Shh-N are necessary to induce a conformational change in the receptor.

4. Discussion

The results obtained in this study demonstrate that the NH$_2$-terminal region of Shh-N is necessary for activation of the receptor but not for binding to the receptor. It has been reported that signalling by hedgehog is transduced by a receptor complex of Ptc, a ligand-binding component, and Smo, a constitutive active signalling component [22,23]. In this multi-component receptor system, the binding of Shh-N to Ptc releases the suppressed signal of Smo. Although the molecular mechanism of the release of the suppression has not been clarified, binding of Shh-N seems to induce a conformational change in Ptc. Recently, it was reported that a palmitic acid modification of Shh-N at a NH$_2$-terminal cysteine residue resulted in enhancing its biological activity without changing its binding affinity to Ptc [33]. In the present study, the low activity of recombinant mouse Shh-N expressed in yeast was caused by a lack of a 10 amino acid peptide at the NH$_2$-terminal of Shh-N, since a mutant Shh-N without a deleted

NH$_2$-terminal region had a comparable activity to Shh-N expressed in L-cells as shown in Fig. 1. Furthermore, desN-Shh-N showed the complete loss of the activity of Shh-N and in addition, antagonistic activity against Shh-N, indicating the ability to bind a receptor remained (Fig. 4). These observations indicate that a certain structure involved in the NH$_2$-terminal region is necessary for Ptc to induce a conformational change for releasing the suppression of Smo signalling. Due to the previously reported crystal structure of murine Shh-N with nine missing amino acid residues [34], we cannot know the actual structure of the protein in our present study. However, we can recognize that the NH$_2$-terminal peptide composed of nine amino acids has a very flexible structure because four glycine residues, conserved in proteins of the hedgehog family, are arranged alternating in this peptide. This flexibility might play a part in inducing a conformational change in Ptc.

The NH$_2$-terminal region of soluble Shh-N seems to be especially susceptible to trypsin-like protease as shown in Fig. 4. Little, besides desN-Shh-N by trypsin digestion, was produced even though there are several expected proteolytic cleavage sites. Various proteases, plasmin, thrombin or tissue plasminogen activator, in vivo might cleave this portion of Shh-N. Actually, crystal Shh-N, lacking the NH$_2$-terminal region, was cleaved between Lys-33 and Arg-34 by thrombin [34]. This provides evidence for the idea that the action of soluble Shh-N diminishes rapidly by digestion of the NH$_2$-terminal region after secretion in vivo because the desN-Shh-N might become an inactive molecule and in addition antagonistic to Shh-N. It has been thought that hedgehog undergoes a lipophilic modification by autoprocessing to retain at the cell surface and that some action of Shh-N is necessary to assist the localized long term event naturally, since a large amount of diffused Shh-N causes embryonic mispatterning [27]. In order to incomplete the lipid modification after complete truncation by autoprocessing of Shh, the soluble Shh-N seems to be produced in vivo. Therefore, further experimentation on our findings from this work might prove meaningful as a clearance mechanism for soluble Shh-N.

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